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(54) Title: CULTURE MEDIA FOR *IN VITRO* FERTILIZATION AND EMBRYO TRANSFER

(57) Abstract

A culture medium for *in vitro* fertilization of human oocytes which comprises: NaCl (96.5 - 106.7 mM) KCl(4.46 - 4.92 mM) $MgSO_4.7H_2O$ (0.18 - 0.22 mM) KH_2PO_4 (0.35 - 0.39 mM) $CaCl_2.2H_2O$ (1.94 - 2.14 mM) $NaHCO_3$ (23.7 - 26.3 mM) Glucose (2.64 - 2.92 mM) Sodium Pyruvate (0.31 - 0.35 mM) Sodium Lactate (20.3 - 22.5 mM) Penicillin (95 - 105 units/ml) Phenol red (5 - 15 micrograms/ml). Culture media wherein the ratio of sodium ions to potassium ions is in the range of from 28 to 32 are preferred.

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CULTURE MEDIA FOR IN VITRO FERTILIZATION AND EMBRYO TRANSFER

The invention relates to culture media and more particularly to culture media for in vitro fertilization and embryo transfer.

The process of in vitro fertilization of human oocytes, cleavage of embryos and embryo transfer require that a culture medium be used to support the embryo for a period of up to three or four days during the various processes necessary for fertilization and early incubation before embryo transfer and reimplantation.

In the natural process of fertilization for a human oocyte the oocyte is supported within the mother within a fluid known as human tubal fluid and it is the object of the present invention to provide a culture medium as a synthetic human tubal fluid.

Approximation of the culture conditions as close as possible to those found in the natural environment of the gametes may be most likely to yield the best results. Using this rationale early workers have formulated a culture media similar in biochemical composition to human tubal fluid with varying rates of success. Examples of these include Tyrodes Medium T6, WM1 (Hoppe and Pitts), Modified Earles, and Hams F10.

One important characteristic of synthetic human tubal fluids appears to the ratio of sodium ions to potassium ions. For natural human tubal fluid this valve is approximately 18. Earlier attempts such as Tyrodes Medium T6 have a value of over 100. We have found that values in between these are most advantageous.

In the present invention we have devised a synthetic culture medium which is believed to approximate human tubal fluid but with desirable additional components and variations in the actual composition, including ratios of concentrations of sodium ions to potassium ions.

In one form therefore the present invention may be said to reside in a culture medium for in vitro fertilization and embryo transfer comprising the following compounds in the following ranges of concentration;

Sodium chloride (NaCl)	96.5 - 106.7 mM
Potassium chloride (KCl)	4.46 - 4.92mM
Magnesium sulphate ($MgSO_47H_2O$)	0.18 - 0.22mM
Potassium phosphate monobasic (KH_2PO_4)	0.35 - 0.39mM
Calcium chloride 2 hydrate ($CaCl_22H_2O$)	1.94 - 2.14mM
Sodium bicarbonate ($NaHCO_3$)	23.7 - 26.3mM
Glucose	2.64 - 2.92mM
Sodium Pyruvate	0.31 - 0.35mM
Sodium Lactate	20.3 - 22.5mM
Penicillin	95 - 105 units/ml
Phenol red	5 - 15 micrograms/ml

In a preferred embodiment of the invention, the ratio of sodium ion concentration to potassium ion concentration is in the range from 28 to 32.

In a further preferred embodiment the ratio of concentrations of sodium ions to potassium ions is 29.3.

In one preferred embodiment of the invention the synthetic human tubal fluid may have the following composition;

Sodium Chloride (NaCl)	101.6 mM
Potassium chloride (KCl)	4.69 mM
Magnesium sulphate ($MgSO_47H_2O$)	0.20mM
Potassium phosphate monobasic (KH_2PO_4)	0.37mM
Calcium chloride 2 hydrate ($CaCl_22H_2O$)	2.04mM
Sodium bicarbonate ($NaHCO_3$)	25.0mM
Glucose	2.78mM

Sodium pyruvate	0.33mM
Sodium lactate	21.4mM
Penicillin	100 units/ml
Phenol red	10 micrograms/ml

In another form the invention may be said to reside in a method of assisting with the in vitro fertilization of human oocytes including the step of handling the human oocytes in a culture medium, the culture medium being comprised of the compounds listed below in the range of compositions listed as follows;

Sodium chloride (NaCl)	96.5 - 106.7mM
Potassium chloride (KCl)	4.46 - 4.92mM
Magnesium sulphate ($MgSO_4$ $7H_2O$)	0.18 - 0.22mM
Potassium phosphate monobasic (KH_2PO_4)	0.35 - 0.39mM
Calcium chloride 2 hydrate ($CaCl_2$ $2H_2O$)	1.94 - 2.14mM
Sodium bicarbonate ($NaHCO_3$)	23.7 - 26.3mM
Glucose	2.64 - 2.92mM
Sodium pyruvate	0.31 - 0.35mM
Sodium lactate	20.3 - 22.5mM
Penicillin	95 - 105 units/ml
Phenol red	5 - 15 micrograms/ml

In a preferred embodiment of this method of the invention, the ratio of sodium ion concentration to potassium ion concentration is in the range of from 28 to 32.

In a further preferred form of the invention, the ratio concentration of sodium ions to potassium ions is 29.3.

In a further preferred form of the invention, the method includes the step of handling the oocytes in a culture medium comprising;

Sodium Chloride (NaCl)	101.6 mM

Potassium chloride (Kcl)	4.69 mM
Magnesium sulphate ($MgSO_4 7H_2O$)	0.20mM
Potassium phosphate monobasic (KH_2PO_4)	0.37mM
Calcium chloride 2 hydrate ($CaCl_2 2H_2O$)	2.04mM
Sodium bicarbonate ($NaHCO_3$)	25.0mM
Glucose	2.78mM
Sodium pyruvate	0.33mM
Sodium lactate	21.4mM
Penicillin	100 units/ml
Phenol red	10 micrograms/ml

In a further form the invention may be said to reside in a culture medium for the in vitro fertilization of human oocytes including sodium potassium ions wherein the ratio of sodium ions to potassium ions is in the range of from 28 to 32.

In a preferred embodiment of this form of the invention, the ratio of sodium ions to potassium ions is 29.3.

This then generally describes the nature of the present invention and it will be seen that by this invention there is provided a culture medium which is not exactly the same as natural human tubal fluid, but which is capable of supporting in vitro fertilization.

To more clearly assist with the understanding of this invention reference will now be made to a preferred embodiment and tests to determine the efficacy of the preferred embodiment.

In one preferred embodiment synthetic human tubal fluid culture medium is as given in Table 1 below (marked synthetic HTF).

The medium may be prepared by using rainwater which has been distilled in glass six times. The bicarbonate-buffered medium is gassed for a minimum of five minutes with humidified 5% oxygen 5% carbon dioxide

90% nitrogen mixture and sterilized by passage through a 0.45-0.2 micrometre filter membrane (Millipore, Sydney, Australia or Amicon Sterilet Adelaide, Australia) and then stored at 4°C for up to two weeks before use. A minimum of six hours or preferably the day before being used the bicarbonate buffered medium is gassed again for two to three minutes with the same gas mixture as above and the protein component is added.

In a similar way a known culture medium Tyrodes Medium T6 having a composition as given in Table 1 below was also prepared.

TABLE 1

COMPONENT	SYNTHETIC HFT	T6
NaCl	101.6	99.4
KCl	4.69	1.42
$MgSO_4.7H_2O$	0.20	0.71
KH_2PO_4	0.37	-
$CaCl_2.2H_2O$	2.04	1.78
$NaHCO_3$	25	25
Glucose	2.78	5.56
Na pyruvate	0.33	0.47
Na lactate	21.4	24.9
Penicillin	100 U/ml	100 U/ml
Streptomycin SO_4	-	50 ug/ml
Phenol red	0.001% (w/v)	0.001% (w/v)

Tests have been carried out using both mouse embryo development in vitro and with initiation of human pregnancy in an endeavour to discover which components of the T6 medium and the synthetic human tubal fluid according to this invention might be responsible for observed differences in mouse embryo development in vitro and initiation of human pregnancies. The results show that for human pregnancy initiation almost three times as many pregnancies occurred when fertilization and culture were carried out

using the synthetic human tubal fluid of the present invention over the T6 medium.

In comparison of the two compositions a greatest difference in composition of the two media are their Na^+/K^+ ratios. We refer to these as sodium/potassium for the rest of the specification.

The ratios of concentrations of sodium ions to potassium ions for these are as follows:

Synthetic Human Tubal Fluid according to the present invention	-	29.3
T6	-	105

When media are tested with sodium/potassium levels varying from 150.5/1.42 millimoles to 148.2/5.06 millimoles results showed that there was significant linear and quadratic responses in the number of embryos developing to expanded blastocysts with increasing levels of K^+.

In medium containing the potassium levels of T6 medium 75% of the zygotes developed which was significantly fewer than the 95-100% embryos developing when the potassium level was 2.3 to 5.1 millimoles which is the range for the synthetic human tubal fluid of the present invention.

The greatest number of mouse zygotes developing to expanded blastocysts when cultured in synthetic human tubal fluid medium of the present invention compared to T6 medium was paralleled by the three fold increase of the number of pregnancies initiated in those patients whose gametes had been fertilized and cultured in the medium in the present invention rather than T6 medium.

The present invention therefore provides a synthetic human tubal fluid culture medium which is more than just a direct replication of naturally occurring human tubal fluid but has enhanced viability.

Potassium chloride (Kcl)	4.69 mM
Magnesium sulphate ($MgSO_47H_2O$)	0.20mM
Potassium phosphate monobasic (KH_2PO_4)	0.37mM
Calcium chloride 2 hydrate ($CaCl_22H_2O$)	2.04mM
Sodium bicarbonate ($NaHCO_3$)	25.0mM
Glucose	2.78mM
Sodium pyruvate	0.33mM
Sodium lactate	21.4mM
Penicillin	100 units/ml
Phenol red	10 micrograms/ml

In a further form the invention may be said to reside in a culture medium for the in vitro fertilization of human oocytes including sodium potassium ions wherein the ratio of sodium ions to potassium ions is in the range of from 28 to 32.

In a preferred embodiment of this form of the invention, the ratio of sodium ions to potassium ions is 29.3.

This then generally describes the nature of the present invention and it will be seen that by this invention there is provided a culture medium which is not exactly the same as natural human tubal fluid, but which is capable of supporting in vitro fertilization.

To more clearly assist with the understanding of this invention reference will now be made to a preferred embodiment and tests to determine the efficacy of the preferred embodiment.

In one preferred embodiment synthetic human tubal fluid culture medium is as given in Table 1 below (marked synthetic HTF).

The medium may be prepared by using rainwater which has been distilled in glass six times. The bicarbonate-buffered medium is gassed for a minimum of five minutes with humidified 5% oxygen 5% carbon dioxide

90% nitrogen mixture and sterilized by passage through a 0.45-0.2 micrometre filter membrane (Millipore, Sydney, Australia or Amicon Sterilet Adelaide, Australia) and then stored at 4^0C for up to two weeks before use. A minimum of six hours or preferably the day before being used the bicarbonate buffered medium is gassed again for two to three minutes with the same gas mixture as above and the protein component is added.

In a similar way a known culture medium Tyrodes Medium T6 having a composition as given in Table 1 below was also prepared.

TABLE 1

COMPONENT	SYNTHETIC HFT	T6
NaCl	101.6	99.4
KCl	4.69	1.42
$MgSO_4.7H_2O$	0.20	0.71
KH_2PO_4	0.37	-
$CaCl_2.2H_2O$	2.04	1.78
$NaHCO_3$	25	25
Glucose	2.78	5.56
Na pyruvate	0.33	0.47
Na lactate	21.4	24.9
Penicillin	100 U/ml	100 U/ml
Streptomycin SO_4	-	50 ug/ml
Phenol red	0.001% (w/v)	0.001% (w/v)

Tests have been carried out using both mouse embryo development in vitro and with initiation of human pregnancy in an endeavour to discover which components of the T6 medium and the synthetic human tubal fluid according to this invention might be responsible for observed differences in mouse embryo development in vitro and initiation of human pregnancies. The results show that for human pregnancy initiation almost three times as many pregnancies occurred when fertilization and culture were carried out

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A culture medium for in vitro fertilization of human oocytes comprising:

Sodium chloride (NaCl)	96.5 - 106.7 mM
Potassium chloride (KCl)	4.46 - 4.92mM
Magnesium sulphate ($MgSO_47H_2O$)	0.18 - 0.22mM
Potassium phosphate monobasic (KH_2PO_4)	0.35 - 0.39mM
Calcium chloride 2 hydrate ($CaCl_22H_2O$)	1.94 - 2.14mM
Sodium bicarbonate ($NaHCO_3$)	23.7 - 26.3mM
Glucose	2.64 - 2.92mM
Sodium Pyruvate	0.31 - 0.35mM
Sodium Lactate	20.3 - 22.5mM
Penicillin	95 - 105 units/ml
Phenol red	5 - 15 micrograms/ml

2. A culture medium in Claim 1 wherein the ratio of sodium ion concentration to potassium ion concentration is in the range of from 28 to 32.

3. A culture medium as in Claim 2 wherein the ratio of concentrations of sodium ions to potassium ions is approximately 29.3.

4. A culture medium as in Claim 1 comprising approximately;

Sodium Chloride (NaCl)	101.6 mM
Potassium chloride (KCl)	4.69 mM
Magnesium sulphate ($MgSO_47H_2O$)	0.20mM
Potassium phosphate monobasic (KH_2PO_4)	0.37mM
Calcium chloride 2 hydrate ($CaCl_22H_2O$)	2.04mM
Sodium bicarbonate ($NaHCO_3$)	25.0mM
Glucose	2.78mM
Sodium pyruvate	0.33mM

Sodium lactate	21.4mM
Penicillin	100 units/ml
Phenol red	10 micrograms/ml

5. A method of assisting with the in vitro fertilization of human oocytes including the steps of handling the oocytes in a culture medium, the culture medium being of the compounds being listed below in the range of compositions as follows;

Sodium chloride (NaCl)	96.5 - 106.7 mM
Potassium chloride (KCl)	4.46 - 4.92mM
Magnesium sulphate ($MgSO_47H_2O$)	0.18 - 0.22mM
Potassium phosphate monobasic (KH_2PO_4)	0.35 - 0.39mM
Calcium chloride 2 hydrate ($CaC1_22H_2O$)	1.94 - 2.14mM
Sodium bicarbonate ($NaHCO_3$)	23.7 - 26.3mM
Glucose	2.64 - 2.92mM
Sodium Pyruvate	0.31 - 0.35mM
Sodium Lactate	20.3 - 22.5mM
Penicillin	95 - 105 units/ml
Phenol red	5 - 15 micrograms/ml

6. A method as in Claim 5 wherein the ratio of sodium ion concentration to potassium ion concentration is in the range of from 28 to 32.

7. A method as in Claim 6 wherein the ratio of concentration of sodium ions to potassium ions is approximately 29.3.

8. A method as in Claim 5 wherein the culture medium has a concentration as follows;

Sodium Chloride (NaCl)	101.6 mM
Potassium chloride (Kcl)	4.69 mM
Magnesium sulphate ($MgSO_47H_2O$)	0.20mM
Potassium phosphate monobasic (KH_2PO_4)	0.37mM

Calcium chloride 2 hydrate ($CaCl_2 2H_2O$)	2.04mM
Sodium bicarbonate ($NaHCO_3$)	25.0mM
Glucose	2.78mM
Sodium pyruvate	0.33mM
Sodium lactate	21.4mM
Penicillin	100 units/ml
Phenol red	10 micrograms/ml

9. A culture medium for the in vitro fertilization of human oocytes including sodium and potassium ions wherein the ratio of sodium ions to potassium ions is in the range of from 28 to 32.

10. A culture medium as in Claim 9 wherein the ratio of sodium ions to potassium ions is approximately 29.3.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 86/00170

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C12N 5/00, 5/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C12N 5/00, 5/02

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU : IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No [13]
P,X	Fertility and Sterility, Volume 44, No.4, issued October 1985, Quinn et al, "Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid', see pages 493-498	1-10
A	'In Vitro Fertilization and Embryo Transfer', Edited by A. Trounson and C. Wood, published 1984 (Churchill Livingstone) pages 32-33, 119-120	1-10
A	'Clinical In Vitro Fertilization', Edited by A. Trounson and C. Wood, published 1984 (Springer-Verlag, Berlin) pages 104-105	1-10
A	'Human Conception in Vitro', Edited by R.G. Edwards and J.M. Purdy, published 1982 (Academic Press Inc., London) pages 138-139, 214	1-10
P,X	WO,A, 86/00335 (VEECH) 16 January 1986 (16.01.86)	9-10
X	US,A, 4473647 (CARPENTER et al) 25 September 1984 (25.09.84) See column 8 lines 57063	9-10
X	US,A, 4443432 (GARABEDIAN et al) 17 April 1984 (17.04.84) See column 4 lines 25-28	9-10

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 September 1986 (19.09.86)	29 SEP 1986
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	Gordon Masters G. MASTERS

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 86/00170

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
US 4473647	EP 72864	AU 83380/82	WO 8202900
US 4443432	AU 88532/82	CA 1187799	DK 4417/82
	EP 76658	JP 58072515	ZA 8206859
WO 8600335	AU 45402/85	EP 188529	

END OF ANNEX